MEMORANDUM

To: Steve Willis (UXO Pro)

From: Eleanor M. Jennings

Date: September 12, 2018

Subject: Assessment of available, re-baseline microbial data
Williams AFB, ST012

cc:

This technical memo is being submitted to summarize and discuss the microbial re-baseline data provided to date by the USAF.

1 BACKGROUND

Microbial data was obtained by the USAF and its contractors as part of a re-baseline assessment, defined as analyses performed after the ending of SEE for the purpose of discerning current biogeochemical conditions. This data was presented during the 6/28/18 BCT meeting. Much of the data eluded to during the 8/23/18 BCT meeting was not presented at that time. However, the referenced data report from Microbial Insights, Inc. was subsequently provided by the USAF contractors for an independent assessment.

The goal of this technical memo is to evaluate and interpret these data. Remaining data gaps will be identified and suggested future actions will be discussed.

1.1 Report Organization

To facilitate review, this technical memo is organized into the following sections:

- Section 1: Background, including a review of the microbial assessments proposed in the MNA checklist
- Section 2: Summary and assessment of currently available microbial analyses
- Section 3: Identified data gaps
- Section 4: Suggested path forward
- Section 5: Summary
- Table 1: Data Summary

- Appendix A: Technical description of the direct microbial analyses performed or proposed
- Appendix B: Microbial Insights Data Report 092PE

1.2 Review of Microbial Assessments Proposed in MNA Checklist

As part of the EPA Technical Network Checklist ("Checklist"; June 2017), an indirect assessment of the indigenous microbial community was proposed through the evaluation of groundwater geochemical parameters. In addition, direct evaluations of the microbial community were also proposed for the purpose of confirming key metabolic activities, including in-situ benzene biodegradation.

1.2.1 Indirect Geochemical Assessments Proposed

Multiple, and specific, groundwater geochemical parameters were requested in the Checklist. The purpose of these parameters was to provide an inexpensive, indirect way of evaluating the activity of a potential indigenous benzene biodegrading population.

Some of these metrics were already being obtained by the USAF contractors. Other metrics were promised as part of future assessments, in documents such as the decision matrix and Table 5.1 of the EBR Workplan. Finally, the Checklist also requested some geochemical parameters not previously tested or promised.

1.2.2 Direct Microbial Assessments Proposed, Using Molecular and Stable Isotope Technologies

As part of the Checklist, a task-specific direct microbial assessment was requested. The analysis desired is a stable-isotope, in-situ microbial sampler (Bio-Trap®) evaluation. A full description of this technology is provided in **Appendix A**. The primary benefit of this assessment is that it can provide absolute proof of in-situ benzene (for example) biodegradation under current, in-situ conditions. This is possible because the samplers are baited with an isotopically-labeled form of a target compound (such as benzene) prior to being deployed in a target monitoring well. This isotopically-labeled compound is consumed by indigenous degrading microbes, and the isotopic tag becomes incorporated into the degrading microbes' biomass. The only way that this isotopic tag can be found in this biomass is if the cell consumed the isotopically-tagged target material. Thus, this finding would be irrefutable proof of the bioattenuation of the target compound under current, in-situ conditions. In addition, the degree of isotopic-tag incorporation is quantified.

This technology was recommended because it is the only method for definitively proving if in-situ benzene bioattenuation is currently occurring in an environment. The benefits of this technology assume, however, that the Bio-Trap® samplers are used in this manner, as it was designed.

2 SUMMARY AND ASSESSMENT OF CURRENTLY AVAILABLE MICROBIAL ANALYSIS DATA

The re-baseline data obtained by the USAF and its contractors includes both indirect geochemical assessments as well as direct microbial analyses.

2.1 Indirect Geochemical Assessments Conducted

The biogeochemical parameters requested in the Checklist were chosen in order to provide adequate information to indirectly monitor microbial activity at the Site. Although not as accurate or refined as direct microbial evaluations, this information provides an inexpensive and easy method for monitoring conditions that are key to successful bioremediation.

Available geochemical data presented during the monthly BCT meetings includes LNAPL presence and distribution, dissolved benzene concentrations and distribution, and groundwater arsenic concentrations. Some iron speciation data has recently been presented. However, this is not enough information to indirectly evaluate or monitor conditions associated with the bioremediation of hydrocarbons.

In comparison to the Checklist, the following baseline geochemistry parameters are either not being tested or are not being reported:

Temperature*	Phosphorous
pH*	Hydrogen Sulfide
ORP*	Alkalinity
DO*	

* USAF agreed to analyze these (ROD-Amendment Table 5.1; USAF Flowchart)

This data is important, as current geochemical conditions need to be compared to the below-presented biological data in order to better understand exactly what the indigenous microbial population is capable of doing (from a biodegradation perspective) under current conditions. This information becomes the basis for the MNA or EBR stage of a project.

2.2 Direct Microbial Assessments Conducted

Direct microbial assessments were first reported during the 6/28/18 BCT meeting.

2.2.1 Initial qPCR performed using Bio-Trap® sampler

The initial, direct microbial assessment data was presented as slides in the 6/28/18 BCT meeting. The associated laboratory report was not provided. This microbial data consisted of qPCR data (**Table 1**) obtained from the use of Bio-Trap® in-situ microcosm samplers (see **Appendix A** for a technology description). This is not a standard method for obtaining groundwater samples for a common qPCR analysis.

Although Bio-Trap® samplers have been used to capture and subsequently evaluate indigenous microbial communities, this is a process normally reserved only for microbial

communities of a very small population size (a fact that was confirmed by a call with the manufacturer [Microbial Insights] on 5/7/18). This is because the Bio-Traps® are known to artificially inflate the reported population sizes found in an environment, in comparison to the more standard practice of a groundwater grab-sample. Microbial Insights confirmed that the Bio-Traps® inflate an apparent population size by up to multiple orders of magnitude, and this inflation is inconsistent between samplers deployed simultaneously or at various times but in the same location.

As such, Microbial Insights confirmed that any qPCR data resulting from a Bio-Trap® sampler was to be considered qualitative and not quantitative.

The only two genes tested during this initial, qualitative variation of a qPCR were the EBAC and ABS genes, which assess the total eubacterial population and the sulfate-reducing population, respectively. No other genes were tested for. However, the community DNA isolated from the Bio-Trap® samplers was apparently frozen in case of a future need (a customary practice).

At this point, the following pieces of information were known:

- The qualitative microbial assessment performed confirmed the presence of a total eubacterial population in all targeted monitoring wells, and the presence of a sulfate-reducing population in three of the six tested monitoring wells (**Table 1**);
- Indigenous benzene degraders were not yet evaluated, as only the total eubacterial population and sulfate-reducer population was qualitatively assessed; and
- Because the Bio-Traps were not used in a standard way (which would include baiting the samplers with isotopically-labeled benzene), actual in-situ biodegradation under current geochemical conditions was not assessed.

When asked about this issue during the 6/28/18 BCT meeting, the USAF and its contractors stated that the Bio-Traps® were used per the Checklist instructions. ADEQ then explained that the Checklist asked for the standard Bio-Trap® procedure be followed. This included the use of the isotopically-labeled benzene in order to confirm in-situ benzene biodegradation under current conditions as well as to provide a current benzene biodegradation rate.

In response, the USAF and its contractors stated that they would return to the same monitoring wells and pull grab samples of groundwater for a comparative qPCR assessment. ADEQ suggested to the USAF and their contractor that an analysis for actual benzene biodegradation be included at the same time. That data has not yet been reported.

2.2.2 *Follow up Quant-Array (Petroleum) assessment performed using frozen community DNA obtained from Bio-Trap® samplers*

During the 8/23/18 BCT meeting, it was revealed that the USAF and its contractor had conducted a follow up microbial analysis. The raw data report was provided, as requested, and assessed (**Table 1**). The follow up microbial analysis, also performed by Microbial

Insights, was the Quant-Array (Petroleum) assessment. This is a technology that is capable of quantifying 22 pre-determined microbial groups or metabolic capabilities that are commonly associated with petroleum-impacted locations (see **Appendix A** for technology details).

It is important to note that this Quant-Array (Petroleum) assessment was performed on the frozen community DNA isolated from the Bio-Trap® samplers initially used for the above-described qPCR assessments. A much more standard method of obtaining community DNA is through a groundwater grab sample. As such, the same issue of artificial result inflation will apply to the Quant-Array (Petroleum) data.

2.2.3 *Data assessment*

Total Eubacterial Bacteria and Sulfate-Reducing Bacteria Genes:

Because the Quant-Array (Petroleum) assessment was performed on the frozen community DNA initially isolated from the Bio-Trap® samplers used for the above-described (but much more limited in scope) qPCR assessments, the EBAC and APS gene count results are identical for these two analyses.

All six samples reported the presence of a eubacterial population above detection levels (**Table 1**). As measured by the EBAC gene, this population ranged in size from 3.82×10^2 to 2.21×10^7 cells per bead (cpb). The "cpb" nomenclature reflects that these population sizes are the result of a community grown inside the in-situ samplers and are not taken directly from groundwater. Thus, the above-described issue of population size inflation should be applied. The below detection limit for all genes analyzed in this assay is 2.50×10^2 cpb.

Sulfate-reducing bacteria (as detected by the APS gene) were detected in three of the six samples (**Table 1**):

- ST012-CZ20-051718,
- ST012-LSZ42-051718, and
- ST012-UWBZ24-51718.

Only one of these three samples, ST012-CZ20-051718, also tested positive for genes associated with the biodegradation of aromatic hydrocarbons. The significance of this will be discussed below.

Genes Associated with Aromatic Hydrocarbon Biodegradation:

As a part of the 22 Quant-Array (Petroleum) genes evaluated, a total of seven analyzed genes are potentially involved with benzene bioattenuation (**Table 1**). Only four of these genes were detected, in any samples, however. All of these detections were in a total of two of the six samples: ST012-CZ20-051718 and ST012-LSZ10-0517-18.

Condensed Version of **Table 1**, Focusing on Genes Possibly Associated with Benzene Biodegradation

Gene	Name	Anaerobic or Aerobic?	Description	ST012-CZ20-051718	ST012- LSZ42-051718	ST012-UWBZ31-51718	ST012-LSZ10-051718	ST012-UWBZ24-51718	ST012-CZ02-051718
TOD	toluene/benzene dioxygenase	aerobic	Opens aromatic ring for toluene, benzene, and xylenes	4.9 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2
PHE	phenol hydroxylase	aerobic	Adds oxygen group to the ring of benzene, toluene, xylenes	2.75 e3	<2.5 e2	<2.5 e2	7.99 e2	<2.5 e2	<2.5 e2
RDEG	toluene 2 monooxygenase / phonol hydroxylase	aerobic	Adds oxygen group to the ring of benzene, toluene, xylenes	1.38 e5	<2.5 e2	<2.5 e2	3.31 e3	<2.5 e2	<2.5 e2
RMO	toluene ring hydroylating monooxygenases	aerobic	Adds oxygen group to the ring of benzene, toluene, xylenes	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2
BPH4	Biphenyl/ isopropylbenzene dioxygenase	aerobic	Typically associated with oxygen addition to PCBs, but also associated with a single species of benzene/ isopropylbenzene biodegrading microbe	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2
BCR	Benzoyl Coenzyme A Reductase	anaerobic	Involved in a key step of benzene biodegradation, as well as the biodegradaiton of other aromatic compounds	4.57 e3	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2
ABC	Benzene Carboxylase	anaerobic	The gene that initiates the only known anaerobic benzene biodegradaton pathway	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2

It is important to note that of the hydrocarbon-degradation genes analyzed, only the ABC gene is benzene-specific. The remaining genes are involved in benzene metabolism, but they are also involved in the biodegradation of other aromatic hydrocarbons (such as toluene or the xylenes) or their derivatives. Thus, detections of these genes would be evidence of aromatic hydrocarbon biodegradation, but only the ABC gene can confirm benzene-specific biodegradation.

No sample reported the ABC gene being present at or above the detection limit. This is significant, as the ABC gene codes for the reaction that initiates the only known anaerobic metabolic pathway to biodegrade benzene. Its absence strongly suggests the absence of anaerobic benzene biodegraders in the six samples tested.

Of the remaining six genes tested for aromatic hydrocarbon biodegradation, the BCR gene is the only one also associated with anaerobic processes. The BCR gene was detected only in one sample, ST012-CZ20-051718 (4.57×10^3 cpb). Although the activity of this gene is associated with benzene biodegradation, it is also associated with the biodegradation of other aromatics as well. However, the ABC gene was not detected in the one sample that tested positive for the BCR gene. This suggests that the BCR gene is being used for the anaerobic biodegradation of an aromatic compound other than benzene. If the BCR gene was being used in anaerobic benzene biodegradation, the ABC gene (which is benzene specific, and codes for the initiation step of the single anaerobic benzene biodegradation pathway) should also be present.

This result has further significance because the ST012-CZ20-051718 sample did report a detectable population of sulfate reducers. Thus, although there are sulfate reducers in this sample, and although anaerobic aromatic hydrocarbon biodegradation is occurring in this

sample, benzene is NOT being biodegraded in this sample. As such, not a single test sample demonstrated benzene bioattenuation by possible sulfate-reducing bacteria.

Finally, the above results support the independence of benzene-biodegradation capabilities from the ability to function as a sulfate-reducing bacterium. Simply, if all sulfate-reducers are benzene biodegraders, then the ABC gene for anaerobic benzene biodegradation would have been detected in all samples where sulfate-reducing bacteria were found. This did not occur because these traits are independent of one another.

In regard to genes associated with possible aerobic benzene biodegradation, one sample (ST0-CZ20-051718) reported three at a concentration above detectable levels. However, each of these three genes are also associated with the bioattenuation of aromatic compounds other than benzene, and thus their presence cannot be considered evidence of aerobic benzene biodegradation without additional supporting evidence. Sample ST012-LSZ10-0517-18 reported two of these same three genes at an above detectable level.

One sample (ST0-CZ20-051718) also tested positive for genes associated with the aerobic bioattenuation of either MTBE or TBA as well as the anaerobic biodegradation of longer-chained alkane hydrocarbons. Sample ST012-LSZ10-0517-18 reported positive results for a gene associated with the aerobic biodegradation of naphthalene, phenanthrene, and/or anthracene (**Table 1**).

In summary, the following data were reported by the limited dataset of the Quant-Array (Petroleum) analysis:

- Only two samples reported above-detection level results for genes potentially associated with either aerobic or anaerobic benzene bioattenuation. However, six of these seven genes are also associated with the biodegradation of compounds other than benzene and thus the results do not confirm benzene-specific bioattenuation.
- Only one monitoring well tested positive for any gene possibly associated with benzene biodegradation under anaerobic conditions, however it is believed that this reflects the anaerobic bioattenuation of a non-benzene aromatic compound.
- No anaerobic, benzene-specific, biodegraders were detected in any of the samples, based on levels of the ABC gene.
- No anaerobic benzene biodegradation is occurring in the presence of a sulfate-reducing population. No anaerobic benzene biodegradation appears to be occurring in any of the six samples.
    - This strongly suggests that the addition of sulfate, in an attempt to stimulate benzene-biodegradation under sulfate-reducing (or any other anaerobic set of) conditions, is likely to not be successful at this time with the current microbial population.

- Aerobic benzene biodegradation may possibly be occurring in two of the six samples.

3 IDENTIFIED DATA GAPS

Based on the above, there are a number of identified data gaps at this time.

3.1 Identified Data Gaps

The primary data gap that still exists involves directly monitoring benzene bioattenuation under current, in-situ conditions. Of all of the genes monitored in the above-described assessments, only one is specific to benzene-only bioattenuation. This ABC gene specifically codes for a metabolic step that is required for anaerobic benzene bioattenuation. It was not detected in any of the six samples.

However, because aerobic benzene biodegradation can occur via multiple different pathways, there are multiple possible initiation steps. These initial steps are the same for the metabolism of other aromatic hydrocarbons, however. Additionally, subsequent metabolic steps are also often shared with other aromatic hydrocarbons. Thus, there are no benzene-specific aerobic biodegradation genes available in the Quant-Array (Petroleum) assay. As a result, it is not definitive whether the results of this analysis reflect the occurrence of aerobic benzene biodegradation or not.

The second major data gap is knowing under what environmental condition(s) will benzene biodegradation (if possible) actually occur. The currently available data from this Quant-Array (Petroleum) analysis appears to only support the possibility of benzene biodegradation under aerobic conditions. However, this is only a potential, as benzene-specific biodegradation was not confirmed during this analysis.

A third data gap involves a need to better understand current biogeochemical conditions. The Quant-Array (Petroleum) data reported detectable gene counts for both anaerobic and aerobic processes. The current geochemical condition of the Site needs to be further characterized.

3.2 Importance of Filling Identified Data Gaps

In order to avoid costly delays, and to facilitate progress with in-situ benzene biodegradation, it is critical to confirm if this activity is actually occurring under current conditions. If a benzene biodegrading population is present, then a better understanding needs to be obtained about these microbes in order to stimulate this community. If this population is not present, and thus cannot act as a seed for enhanced bioremediation (EBR), then appropriate plans need to be made for moving forward.

If a benzene-biodegrading population is confirmed to be active under current site conditions, then these current conditions need to be better understood. A strong shift away from conditions supporting biodegradation at the Site (such as a shift from aerobic conditions to

sulfate-reducing conditions) can kill the biodegrading community. This loss may be irreversible.

4 SUGGESTED PATH FORWARD

Given the above-presented results, there are a number of suggestions regarding a path forward. These will help increase the chance of successful in-situ biodegradation of benzene at the Site.

4.1 Suggested Geochemical Analyses

The suggested re-baseline geochemical evaluations and schedule outlined in the Checklist should be followed. The resulting data should be fully analyzed before any injections occur that may alter environmental conditions. In short, there needs to be a full understanding of current biogeochemical conditions.

4.2 Suggested Microbial Analyses

The suggested re-baseline, direct microbial assessments and schedule outlined in the Checklist should be followed. This includes performing a traditional, stable-isotope probe Bio-Trap® assessment. The resulting data should be fully analyzed before any injections occur that may alter microbial-critical environmental conditions. In short, a better understanding of current biogeochemical conditions would be extremely beneficial.

The reason that the traditional Bio-Trap® assessment (which includes the use of the stable-isotope tagging) was proposed is that it is the only mechanism to definitively confirm the biodegradation of benzene under in-situ conditions at this Site. Because the Site is impacted with multiple other aromatic hydrocarbons, it is critical to tease out benzene-specific biodegradation from that of other compounds. Although the above-analyzed Quant-Array (Petroleum) assessment provides very useful information, the results do not definitively confirm in-situ benzene biodegradation under current conditions. Instead, the results show that benzene biodegradation is not occurring through anaerobic pathways, and results are ambiguous regarding any aerobic biodegradation.

In short, the question remains regarding if an indigenous benzene-specific biodegrading population is currently active at the Site. The most direct way to get a definitive answer to this question is to use the technique of stable-isotope probes – in other words, the traditional method of using the Bio-Trap® stable isotope in-situ samplers. Once the presence or absence of a benzene-specific biodegrading microbial population has been answered, then the true potential for either MNA or EBR can begin to be addressed.

If in-situ benzene biodegradation is confirmed to be occurring under current site conditions, then a clear understanding of current geochemical conditions is necessary in order to determine how to increase the effectiveness of the benzene-biodegrading community. If in-

situ benzene biodegradation is found to not be occurring under current conditions, then further assessments should be conducted to determine the best path forward for the Site.

The current Site plan includes the stimulation of benzene biodegradation through the addition of sulfate. However, the results discussed in this document do not support this approach, as the Quant-Array (Petroleum) analysis data does not report any anaerobic benzene metabolism occurring in any of the samples. Therefore, if benzene bioattenuation is confirmed to be occurring under current site conditions, a better understanding of these current conditions needs to be achieved in order to better determine the real potential for either MNA or EBR.

5 SUMMARY

Frozen microbial community DNA, initially obtained for a narrow-spectrum genetic profile of six Site samples, was used for a more broad Quant-Array (Petroleum) genetic analysis. The key results of this available data reveal:

- No samples revealed detectable levels of anaerobic benzene-specific biodegradation.
- Although aerobic biodegradation of aromatic hydrocarbons was detected in two of the six samples, it cannot be confirmed that these results are from benzene-specific bioattenuation or if the results reflect the degradation of other, non-benzene, aromatic hydrocarbons.
- The presence of an indigenous, benzene-specific biodegrading microbial population has not yet been confirmed for this Site.
- Benzene biodegradation under sulfate-reducing conditions is not occurring at this Site.

This data should be strongly considered when future Site MNA or EBR plans are being employed.

Table 1
Williams AFB Ste012
Microbial Data

Gene	Name	Anaerobic or Aerobic?	Description	ST012-CZ20-051718	ST012-LSZ42-051718	ST012-UWBZ31-51718	ST012-LSZ10-051718	ST012-UWBZ24-51718	ST012-CZ02-051718
TOD	toluene/benzene dioxygenase	aerobic	Opens aromatic ring for toluene, benzene, and xylenes	4.9 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2
PHE	phenol hydroxylase	aerobic	Adds oxygen group to the ring of benzene, toluene, xylenes	2.75 e3	<2.5 e2	<2.5 e2	7.99 e2	<2.5 e2	<2.5 e2
RDEG	toluene 2 monooxygenase / phonol hydroxylase	aerobic	Adds oxygen group to the ring of benzene, toluene, xylenes	1.38 e5	<2.5 e2	<2.5 e2	1.31 e3	<2.5 e2	<2.5 e2
RMO	toluene ring hydroylating monooxygenases	aerobic	Adds oxygen group to the ring of benzene, toluene, xylenes	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2
TOL	xylene/toluene monooxygenase	aerobic	Toluene specific, adds oxygen to methyl group	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2
EDO	Ethylbenzene/ isopropylbenzene dioxygenase	aerobic	Adds oxygen to ethylbenzene or isopropylbenzene	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2
BPH4	Biphenyl/ isopropylbenzene dioxygenase	aerobic	Typically associated with oxygen addition to PCBs, but also associated with a single species of benzene/ isopropylbenzene biodegrading microbe	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2
PM1	Methylibium petroleiphilum (PM1)	aerobic	A microbe (PM1) specifically identified with MTBE or TBA biodegradation	2.5 e5	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2
TBA	TBA monooxygenase	aerobic	The TBA-specific biodegradation gene found in microbe PMI	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2
NAH	Naphthalene Dioxygenase	aerobic	Gene that initiates aerobic biodegradation of naphthalene and many other PAHs	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2
NiDA	Naphthalene-inducible Dioxygenase	aerobic	Napthalene specific bioattenuation	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2
PHN	Phenanthrene Dioxygenase	aerobic	Associated with biodegradation of naphthalene, phenanthrene, and anthracene	<2.5 e2	<2.5 e2	<2.5 e2	1.31 e3	<2.5 e2	<2.5 e2
ALK	Alkane Monooxygenase	aerobic	N-alkane (C5-C16) biodegradation	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2
ALMA	Alkane Monooxygenase	aerobic	N-alkane (C20-C32) biodegradation	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2
BCR	Benzoyl Coenzyme A Reductase	anaerobic	Involved in a key step of benzene biodegradation, as well as the biodegradaiton of other aromatic compounds	4.57 e3	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2
BSS	Benzylsuccinate Synthase	anaerobic	Toluene, ethylbenzene, and xylene anaerobic biodegradation	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2
ABC	Benzene Carboxylase	anaerobic	The gene that initiates the only known anaerobic benzene biodegradaton pathway	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2
MNSSA	Naphthylmethylsuccinate Synthase	anaerobic	Similary to BSS gene, for toluene biodegradation	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2
ANC	Naphthalene Carboxylase	anaerobic	Essential step in anaerobic naphthalene biodegradation	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2
ASSA	Alkylsuccinate Synthase	anaerobic	Involved in n-alkane (C6-C18) biodegradaton under nitrate-reducing or sulfate-reducing conditions	4.2 e3	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2	<2.5 e2
EBAC	Total Eubacteria	Bacterial population size	Total microbial population size	2.21 e7	1.6 e5	1.64 e3	5.10 e6	1.33 e6	3.82 e2
APS	Sulfate Reducing Bacteria	SRB bacterial population size	Total population size of sulfate-reducing bacteria	2.41 e6	6.09 e4	<2.5 e2	<2.5 e2	2.20 e5	<2.5 e2

Table 1
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Microbial Data

Gene	Name	Anaerobic or Aerobic?	Description		ST012-CZ20-051718	ST012-LSZ42-051718	ST012-UWBZ31-51718	ST012-LSZ10-051718	ST012-UWBZ24-51718	ST012-CZ02-051718
Data from initial qPCR Assessment										
EBAC					2.21 e7	1.6 e5	1.64 e3	5.10 e6	1.33 e6	3.82 e2
APS					2.41 e6	6.09 e4	<2.5 e2	<2.5 e2	2.20 e5	<2.5 e2

The initial qPCR data is presented below the blue line, and was initially presented at the 6/28/18 BCT meeting

Quant-Array (Petroleum) assay results are presented above the blue line

This data was obtained through the use of in-situ microcosm samplers. These were initally used to obtain community DNA, which was then subjected to a qPCR for the EBAC and APS genes. This community DNA was frozen, and subsequently re-sampled for the above Micro-Array (Petroleum) assay.

Numbers in YELLOW: Cell or gene counts above the 2.5e2 threshold

Numbers in GREEN: Cell or gene counts possibly related to benzene biodegradation, which are also above the 2.5 e2 threshold

Please note that there is only one gene in this assesment that is benzene-only specific (ABC gene).

Appendix A

Technical Descriptions of the Direct Microbial Analyses Performed or Proposed

In-Site Microcosms (Bio-Traps®)

Bio-Traps® are the proprietary in-situ biological sampler made by Microbial Insights, Inc. (MI). Each is composed of a perforated polyvinyl chloride (PVC) tube, capped off at each end, and filled with a few hundred BioSep™ beads. BioSep™ beads (also proprietary to MI) are each about the size of a baby pea and are composed of about 75% activated carbon and 25% Nomex (the same material from which they make such equipment as fire-fighter hose lines and coats). The Nomex component is important because these beads need to be more than sterilized (which just kills all bacteria introduced by those who handled the beads during manufacturing). Instead, the manufacturing process needs to burn off any biomarkers (e.g., DNA, proteins) that would be left behind on the beads after sterilization. The Nomex component stabilizes the beads as they are baked to the point where they are not only sterile, but all biomarkers have also been burned off.

The sterile beads are then put into a vacuum jar, where sterilized, isotopically-labeled benzene (for example) is vaporized. The isotopic label acts as a "tag" or marker that will be followed through the system. The sterile, labeled benzene (now a gas) is forced by the vacuum chamber into the pore-spaces in these beads, sorbing to the activated carbon portion of the beads. At this point, the porous beads have been baited. The presence of a carbon source (isotopically-labeled benzene), coupled with the high degree of porosity, will attract indigenous microbes to enter the beads once the samplers are put into groundwater, as bacteria prefer to attach to surfaces rather than to be free-floating. These beads are then packed into the sterile Bio-Trap® PVC housing, and the resulting sampler is placed into the targeted monitoring well, where indigenous groundwater microbes will enter the pore spaces and take up residence. Benzene-degraders will start metabolizing the labeled benzene that was sorbed to the beads, and the isotopic label will be subsequently transferred from the benzene into the degrader's biomass.

After a few weeks, the Bio-Trap® is removed and sent back to the MI lab. The precise length of time for a sampler to be held in a monitoring well is dependent on both environmental conditions in the target monitoring well and the particular contaminant being targeted for analysis. After opening the Bio-Trap® PVC housing, half of the beads are sent for geochemical assessments where multiple metrics are measured, including:

- How much isotopically labeled carbon dioxide was captured within the beads (the carbon dioxide being generated from the labeled benzene being fully mineralized during target compound biodegradation); and
- The amount of the labeled benzene remaining in the beads
    - From this, it can be calculated how much benzene was consumed during the deployment timeframe, and thus, an in-situ degradation rate can be calculated.

The other half of the beads, taken from the Bio-Trap® sampler, are subjected to biological testing. These beads are first washed, removing all bacteria that had inhabited the beads. This total community would include potential benzene degraders, non-benzene biodegraders, as well as other non-degraders who were also indigenous to the location and took up residence in the BioSep™ beads during deployment. First, the number of cells containing the isotopic tag (from benzene) are counted. Because the only way this tag could become present in microbial biomass is by the bacteria consuming the tagged benzene, the size of the indigenous benzene-degrading population can be calculated. Finally, the lab also investigates the rest of the indigenous microbial population. In addition to quantifying the size of this total population, certain molecules within the cell membranes of these microbes are analyzed. From this assessment, the following information can be learned:

- A full profile of the major groups that make up the indigenous microbial community (fermenters, anaerobes, proteobacteria, and others);
- The diversity of the overall indigenous community, as this is related to the health of this total community
- How stressed the benzene-degrading microbial community is;
- And if this microbial stress is because of the presence of something toxic, or if the stress is likely due to lacking a required nutrient.

All of the above in-situ microcosm data, biotic and abiotic, is quantitative.

Quantitative Polymerase Chain Reaction

A quantitative polymerase chain reaction (qPCR) analysis is a specialized form of a simple polymerase chain reaction (PCR) assessment.

A standard, or simple, PCR is a laboratory method that amplifies a genetic sample that has been isolated from the environment. The sample itself is not altered during the PCR process. Instead, a series of chemical steps simply multiplies the volume of the initial sample, providing an increased quantity of genetic material that is identical in chemical composition to the original sample. A standard PCR reaction has the capability of determining if a specific type of organism is present in an environment. For example, *Pseudomonas* is a bacterial genus capable of degrading benzene under some environmental conditions. A standard PCR reaction, using a proper molecular probe, can determine if *Pseudomonas* is present in an aqueous or soil sample taken from a location. However, a standard PCR cannot determine the number of the target organisms, such as *Pseudomonas*, that are in an environment. The same is true for genes targeting microbial functions, such as aerobic benzene biodegradation – a standard PCR analysis can qualitatively confirm if a specific gene is present, but the technology cannot quantify the amount of gene copies present in a sample. In summary:

- PCR technology can answer the question of if a particular gene is present in an environment.
- This gene can identify an organism, or it can identify a function.

However, a qPCR assay has been modified such that it can quantify targeted genes. In a qPCR assay, a special molecular probe is created to amplify a specific genetic sequence from your target organism. This probe is added to a test tube which also contains purified DNA that was isolated from an environmental sample. In contrast to a standard PCR, however, as each target genetic sequence is copied or amplified, a discrete amount of a fluorescent marker is released into the test tube. The concentration of this fluorescent marker is continuously measured as the initial sample is replicated. Thus, at the end of the qPCR process, the total amount of fluorescent marker released into the test tube medium from the probe can be used to back calculate to the number of gene copies in the original sample. The number of gene copies is then related to the number of target organisms in the initial sample. Thus, qPCR is a quantitative analysis, as opposed to a qualitative one.

This qPCR technology can be applied to aqueous or soil / sediment media, and samples are obtained by a one-time grab sample that is then shipped overnight to the laboratory. Multiple commercial microbiological laboratories can perform this analysis. The technique can easily be modified to enumerate whole classes of target organisms ("aerobic benzene degraders") or a specific genus or species ("*Desulfovibrio desulfuricans*") in order to meet individual project needs. As such, targeted genes can help identify and quantify specific microbes or specific microbial functions. Each gene target is chosen independently. This technology is highly respected within the scientific peer-reviewed literature, and it has been used all over the world to enumerate target populations in contaminated, uncontaminated, and extreme environments. In summary:

- qPCR technology can quantify the amount of a gene present in a sample.
- This gene can identify an organism, or it can identify a function.

One key fact about both of the above-described PCR technologies (standard or quantified) is that one analysis for each gene target is run at a time. In other words, if a person wanted to investigate five specific genes, they would run five separate analyses.

Quant-Array (Petroleum)

In contrast to the above-described PCR technologies, a Quant-Array analysis can quantify many gene targets simultaneously. Therefore, a Quant-Array analysis can simultaneously test for both identification and functional target genes. By doing this, a larger number of genes can be quantified at one time.

The specific Quant-Array (Petroleum) analysis is an "off the shelf" assessment designed to target and quantify 22 pre-selected genes commonly important to monitoring petroleum-impacted sites. These 22 genes include (but are not limited to) those associated with a variety of aerobic and anaerobic aromatic hydrocarbon-degradation pathways. Additionally, anaerobic benzene-specific biodegradation is quantified along with genes associated with the bioattenuation of polyaromatic and alkane hydrocarbons.

The monitoring of the biodegradation of different classes of hydrocarbons is important, as the simultaneous bioattenuation of these compounds may act in either a complimentary or competitive manner to benzene-specific bioattenuation at sites impacted by jet fuels or other petroleum-based materials. Finally, the total microbial population is quantified as are the number of sulfate-reducing bacteria.

Facility-specific questions that can simultaneously (unlike qPCR, where each question is a separate assessment) be answered by the Quant-Array (Petroleum) technology include:

- Are anaerobic aromatic-hydrocarbon biodegraders present, and if so, what is the population size?
- Are anaerobic benzene-specific degrading bacteria present, and if so, what is the size of the population?
- Are aerobic aromatic-hydrocarbon biodegraders present, and if so, what is the size of their population?
- What is the population size for both aerobic and anaerobic microbial degraders of other classes of hydrocarbons or hydrocarbon-related compounds?
- What is the total eubacterial population size?
- What is the total population size of sulfate-reducing bacteria?
- Are population sizes different between one area of a site and another (such as within and outside of any excavated areas)?
- In regard to monitoring site efforts, has the size of a target population changed as a result of site modification efforts such as a bionutrient augmentation process?
- Is the biodegradation of other site hydrocarbons possibly competing for microbial resources?

10515 Research Drive
Knoxville, TN 37932
Phone: 865.573.8188
Fax: 865.573.8133
Web: www.microbe.com

SITE LOGIC Report

QuantArray®-Petro Study

Contact: Gwen Minnier
Phone: (602) 733-6000

Address: Wood Plc
4600 East Washington Street
Suite 600
Phoenix, AZ 85034

Email: gwen.minnier@woodplc.com

MI Identifier: 092PE

Report Date: 07/11/2018

Project: ST012-EBR Former Williams AFB, 9101110001.MOD7.02

Comments:

The QuantArray®-Petro Approach

Comprehensive evaluation of biodegradation potential at petroleum impacted sites is inherently problematic due to two factors:

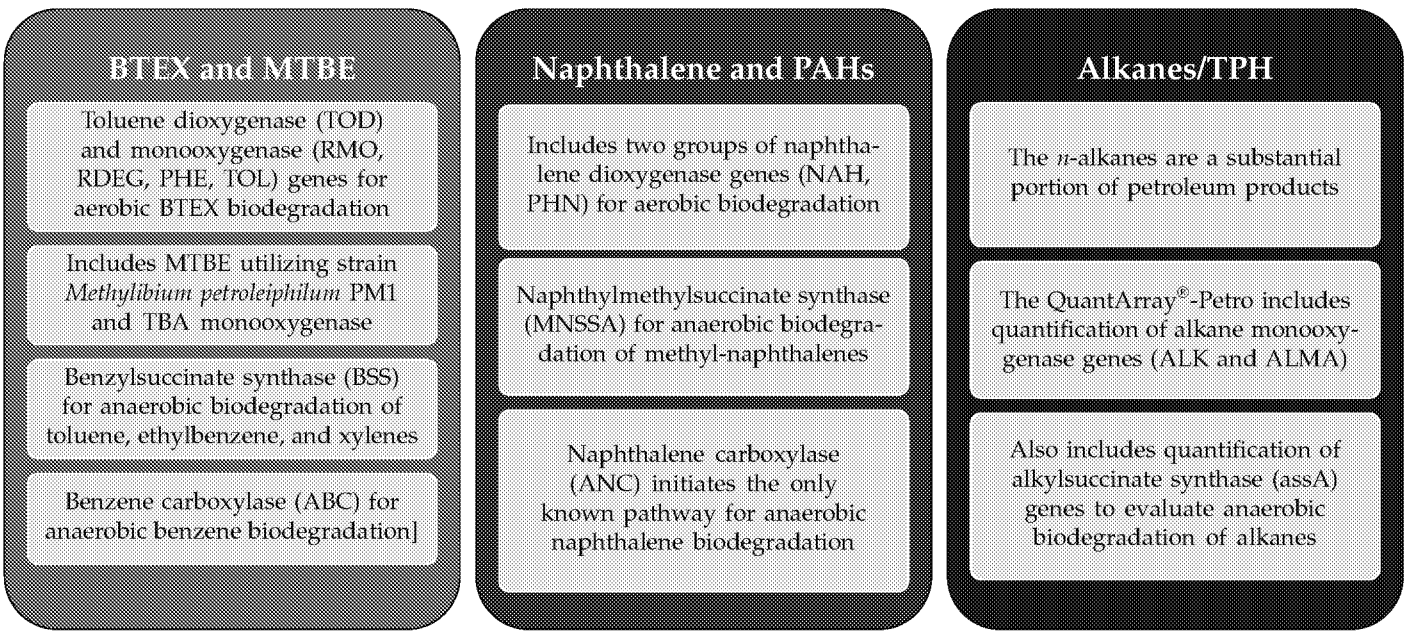
(1) Petroleum products are complex mixtures of hundreds of aliphatic, aromatic, cyclic, and heterocyclic compounds.

(2) Even for common classes of contaminants like benzene, toluene, ethylbenzene, and xylenes (BTEX), biodegradation can proceed by a multitude of pathways.

The QuantArray®-Petro has been designed to address both of these issues by providing the simultaneous quantification of the specific functional genes responsible for both aerobic and anaerobic biodegradation of BTEX, PAHs, and a variety of short and long chain alkanes.

Thus, when combined with chemical and geochemical groundwater monitoring programs, the QuantArray®-Petro allows site managers to simultaneously yet economically evaluate the potential for biodegradation of a spectrum of petroleum hydrocarbons through a multitude of aerobic and anaerobic pathways to give a much clearer and comprehensive view of contaminant biodegradation.

The QuantArray®-Petro is used to quantify specific microorganisms and functional genes to evaluate aerobic and anaerobic biodegradation of the following classes of compounds present in petroleum products:



How do QuantArrays® work?

The QuantArray®-Petro in many respects is a hybrid technology combining the highly parallel detection of microarrays with the accurate and precise quantification provided by qPCR into a single platform. The key to highly parallel qPCR reactions is the nanoliter fluidics platform for low volume, solution phase qPCR reactions.

10515 Research Drive
Knoxville, TN 37932
Phone: 865.573.8188
Fax: 865.573.8133
Web: www.microbe.com

How are QuantArray® results reported?

One of the primary advantages of the QuantArray®-Petro is the simultaneous quantification of a broad spectrum of different microorganisms and key functional genes involved in a variety of pathways for hydrocarbon biodegradation. However, highly parallel quantification combined with various metabolic and cometabolic capabilities of different target organisms can complicate data presentation. Therefore, in addition to Summary Tables, QuantArray®-Petro results will be presented as Microbial Population Summary and Comparison Figures to aid in the data interpretation and subsequent evaluation of site management activities.

Types of Tables and Figures:

Microbial Population Summary	Figure presenting the concentrations of QuantArray®-Petro target gene concentrations (e.g. toluene dioxygenase) relative to typically observed values.
Summary Tables	Tables of target population concentrations grouped by biodegradation pathway and contaminant type.
Comparison Figures	Depending on the project, sample results can be presented to compare changes over time or examine differences in microbial populations along a transect of the dissolved plume.

10515 Research Drive
Knoxville, TN 37932
Phone: 865.573.8188
Fax: 865.573.8133
Web: www.microbe.com

Results

Table 1: Summary of the QuantArray®-Petro results obtained for samples ST012-CZ20-051718, ST012-LSZ42-051718, and ST012-UWBZ31-051718.

Sample Name	ST012-CZ20-051718	ST012-LSZ42-051718	ST012-UWBZ31-051718
Sample Date	05/17/2018	05/17/2018	05/17/2018
Aerobic BTEX and MTBE	cells/bead	cells/bead	cells/bead
Toluene/Benzene Dioxygenase (TOD)	**4.92E+02**	<2.50E+02	<2.50E+02
Phenol Hydroxylase (PHE)	**2.75E+03**	<2.50E+02	<2.50E+02
Toluene 2 Monooxygenase/Phenol Hydroxylase (RDEG)	**1.38E+05**	<2.50E+02	<2.50E+02
Toluene Ring Hydroxylating Monooxygenases (RMO)	<2.50E+02	<2.50E+02	<2.50E+02
Xylene/Toluene Monooxygenase (TOL)	<2.50E+02	<2.50E+02	<2.50E+02
Ethylbenzene/Isopropylbenzene Dioxygenase (EDO)	<2.50E+02	<2.50E+02	<2.50E+02
Biphenyl/Isopropylbenzene Dioxygenase (BPH4)	<2.50E+02	<2.50E+02	<2.50E+02
Methylibium petroleiphilum PM1 (PM1)	**2.50E+05**	<2.50E+02	<2.50E+02
TBA Monooxygenase (TBA)	<2.50E+02	<2.50E+02	<2.50E+02
Aerobic PAHs and Alkanes			
Naphthalene Dioxygenase (NAH)	<2.50E+02	<2.50E+02	<2.50E+02
Naphthalene-inducible Dioxygenase (NidA)	<2.50E+02	<2.50E+02	<2.50E+02
Phenanthrene Dioxygenase (PHN)	<2.50E+02	<2.50E+02	<2.50E+02
Alkane Monooxygenase (ALK)	<2.50E+02	<2.50E+02	<2.50E+02
Alkane Monooxygenase (ALMA)	<2.50E+02	<2.50E+02	<2.50E+02
Anaerobic BTEX			
Benzoyl Coenzyme A Reductase (BCR)	**4.57E+03**	<2.50E+02	<2.50E+02
Benzylsuccinate Synthase (BSS)	<2.50E+02	<2.50E+02	<2.50E+02
Benzene Carboxylase (ABC)	<2.50E+02	<2.50E+02	<2.50E+02
Anaerobic PAHs and Alkanes			
Naphthylmethylsuccinate Synthase (MNSSA)	<2.50E+02	<2.50E+02	<2.50E+02
Naphthalene Carboxylase (ANC)	<2.50E+02	<2.50E+02	<2.50E+02
Alkylsuccinate Synthase (ASSA)	**4.20E+03**	<2.50E+02	<2.50E+02
Other			
Total Eubacteria (EBAC)	**2.21E+07**	**1.60E+05**	**1.64E+03**
Sulfate Reducing Bacteria (APS)	**2.41E+06**	**6.09E+04**	<2.50E+02

Legend:

NA = Not Analyzed
NS = Not Sampled
J = Estimated Gene Copies Below PQL but Above LQL
I = Inhibited
< = Result Not Detected

10515 Research Drive
Knoxville, TN 37932
Phone: 865.573.8188
Fax: 865.573.8133
Web: www.microbe.com

Table 2: Summary of the QuantArray®-Petro results obtained for samples ST012-LSZ10-051718, ST012-UWBZ24-051718, and ST012-CZ02-051718.

Sample Name	ST012-LSZ10-051718	ST012-UWBZ24-051718	ST012-CZ02-051718
Sample Date	**05/17/2018**	**05/17/2018**	**05/17/2018**
Aerobic BTEX and MTBE	cells/bead	cells/bead	cells/bead
Toluene/Benzene Dioxygenase (TOD)	<2.50E+02	<2.50E+02	<2.50E+02
Phenol Hydroxylase (PHE)	**7.99E+02**	<2.50E+02	<2.50E+02
Toluene 2 Monooxygenase/Phenol Hydroxylase (RDEG)	**3.31E+03**	<2.50E+02	<2.50E+02
Toluene Ring Hydroxylating Monooxygenases (RMO)	<2.50E+02	<2.50E+02	<2.50E+02
Xylene/Toluene Monooxygenase (TOL)	<2.50E+02	<2.50E+02	<2.50E+02
Ethylbenzene/Isopropylbenzene Dioxygenase (EDO)	<2.50E+02	<2.50E+02	<2.50E+02
Biphenyl/Isopropylbenzene Dioxygenase (BPH4)	<2.50E+02	<2.50E+02	<2.50E+02
Methylibium petroleiphilum PM1 (PM1)	**1.03E+05**	<2.50E+02	<2.50E+02
TBA Monooxygenase (TBA)	<2.50E+02	<2.50E+02	<2.50E+02
Aerobic PAHs and Alkanes			
Naphthalene Dioxygenase (NAH)	**7.70E+00 (J)**	<2.50E+02	<2.50E+02
Naphthalene-inducible Dioxygenase (NidA)	<2.50E+02	<2.50E+02	<2.50E+02
Phenanthrene Dioxygenase (PHN)	**1.31E+03**	<2.50E+02	<2.50E+02
Alkane Monooxygenase (ALK)	<2.50E+02	<2.50E+02	<2.50E+02
Alkane Monooxygenase (ALMA)	<2.50E+02	<2.50E+02	<2.50E+02
Anaerobic BTEX			
Benzoyl Coenzyme A Reductase (BCR)	<2.50E+02	<2.50E+02	<2.50E+02
Benzylsuccinate Synthase (BSS)	<2.50E+02	<2.50E+02	<2.50E+02
Benzene Carboxylase (ABC)	<2.50E+02	<2.50E+02	<2.50E+02
Anaerobic PAHs and Alkanes			
Naphthylmethylsuccinate Synthase (MNSSA)	<2.50E+02	<2.50E+02	<2.50E+02
Naphthalene Carboxylase (ANC)	<2.50E+02	<2.50E+02	<2.50E+02
Alkylsuccinate Synthase (ASSA)	<2.50E+02	<2.50E+02	<2.50E+02
Other			
Total Eubacteria (EBAC)	**5.10E+06**	**1.33E+06**	**3.82E+02**
Sulfate Reducing Bacteria (APS)	<2.50E+02	**2.20E+05**	<2.50E+02

Legend:

NA = Not Analyzed
NS = Not Sampled
J = Estimated Gene Copies Below PQL but Above LQL
I = Inhibited
< = Result Not Detected

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Knoxville, TN 37932
Phone: 865.573.8188
Fax: 865.573.8133
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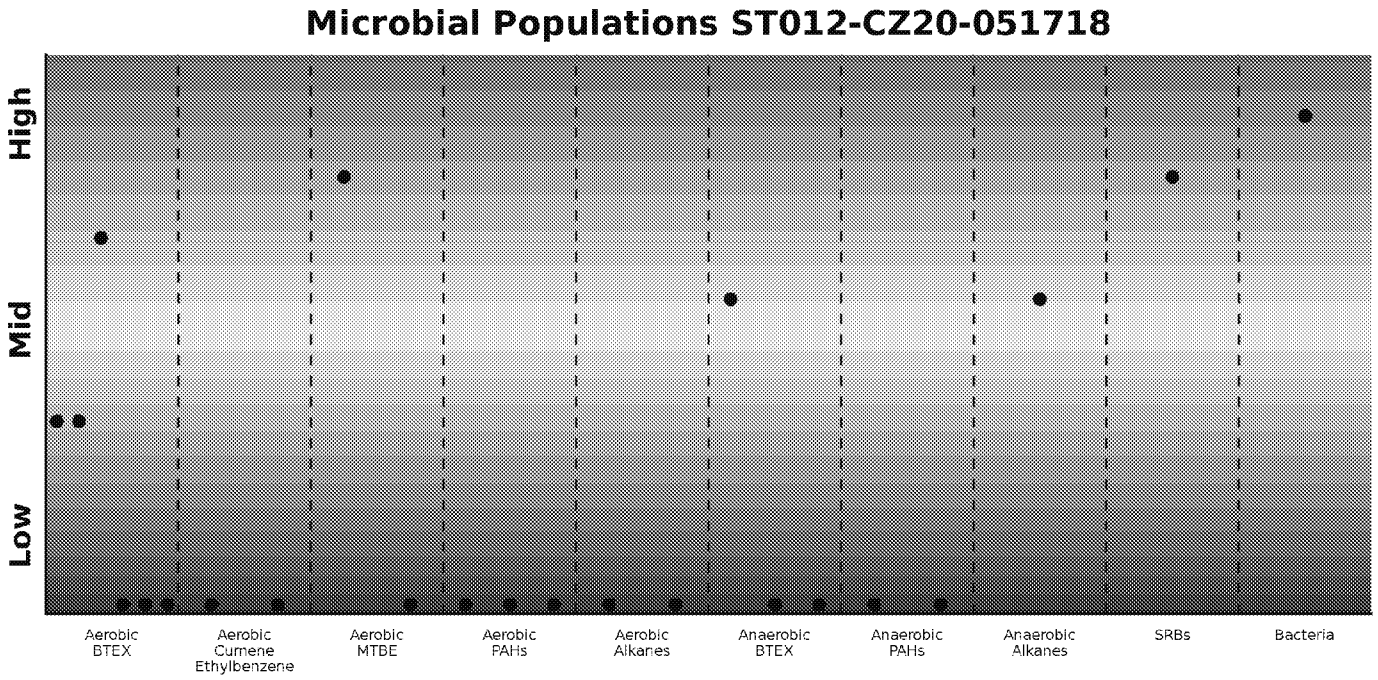

Figure 1: Microbial population summary to aid in evaluating potential pathways and biodegradation of specific contaminants.

Aerobic		Anaerobic	
BTEX	TOD, PHE, RDEG, RMO, TOL, EDO	BTEX	BCR, BSS, ABC
Cumene, Ethylbenzene	EDO, BPH4	Naphthalene/Methylnaphthalene	MNSSA, ANC
MTBE/TBA	PM1, TBA	Alkanes	assA
Naphthalene	NAH, NidA		
Phenanthrene	PHN		
Alkanes	ALK, ALMA		

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Knoxville, TN 37932
Phone: 865.573.8188
Fax: 865.573.8133
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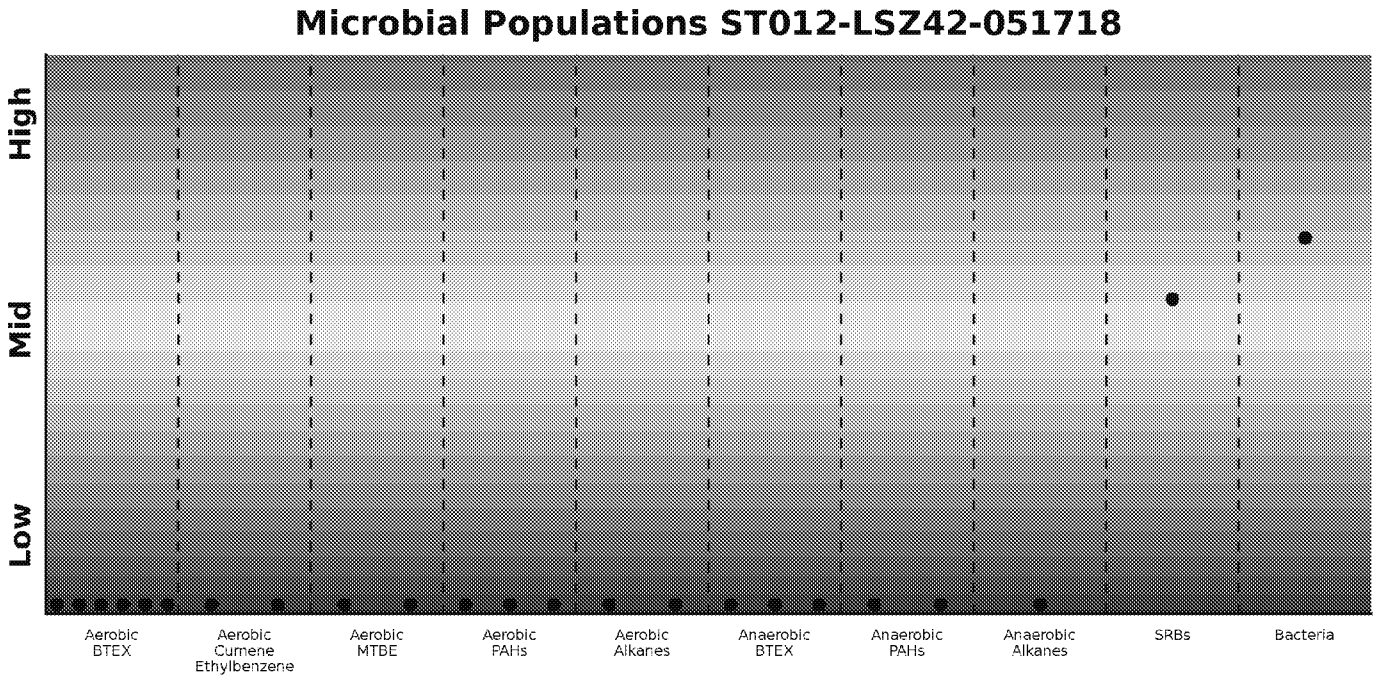

Figure 2: Microbial population summary to aid in evaluating potential pathways and biodegradation of specific contaminants.

Aerobic		Anaerobic	
BTEX	TOD, PHE, RDEG, RMO, TOL, EDO	BTEX	BCR, BSS, ABC
Cumene, Ethylbenzene	EDO, BPH4	Naphthalene/Methylnaphthalene	MNSSA, ANC
MTBE/TBA	PM1, TBA	Alkanes	assA
Naphthalene	NAH, NidA		
Phenanthrene	PHN		
Alkanes	ALK, ALMA		

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Fax: 865.573.8133
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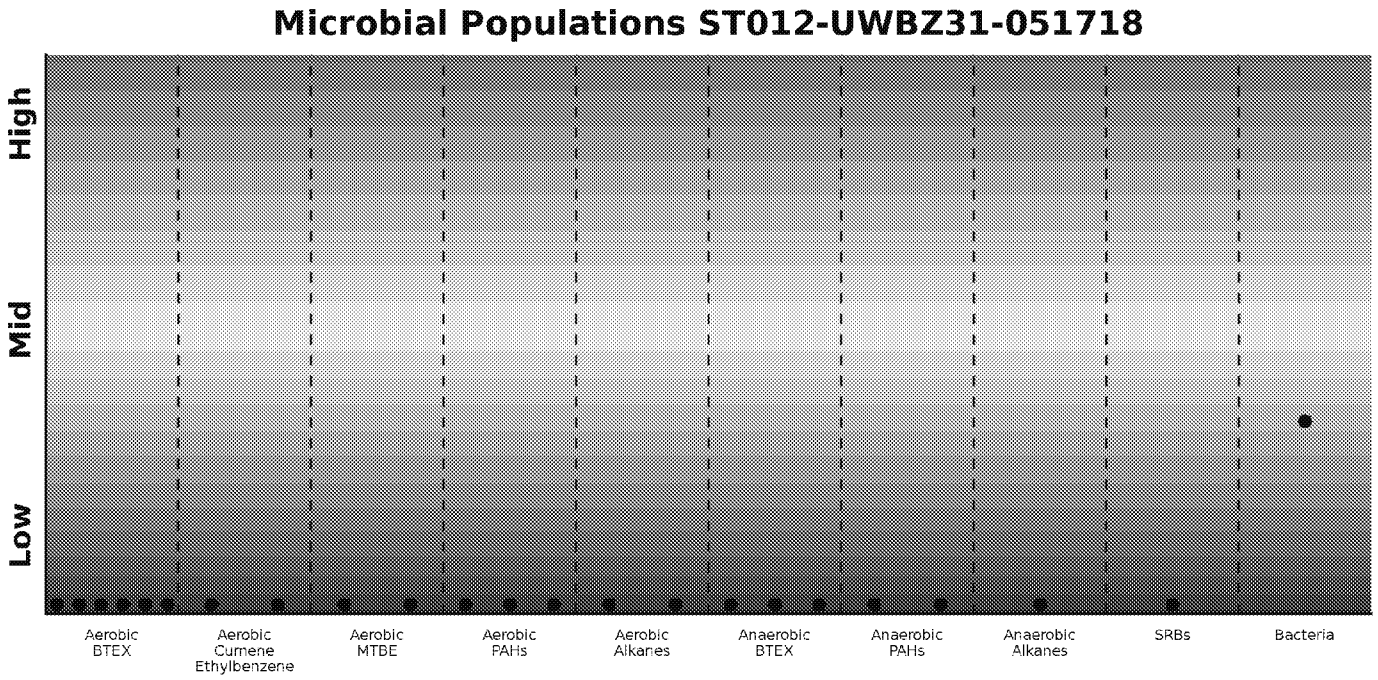

Figure 3: Microbial population summary to aid in evaluating potential pathways and biodegradation of specific contaminants.

Aerobic		Anaerobic	
BTEX	TOD, PHE, RDEG, RMO, TOL, EDO	BTEX	BCR, BSS, ABC
Cumene, Ethylbenzene	EDO, BPH4	Naphthalene/Methylnaphthalene	MNSSA, ANC
MTBE/TBA	PM1, TBA	Alkanes	assA
Naphthalene	NAH, NidA		
Phenanthrene	PHN		
Alkanes	ALK, ALMA		

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Fax: 865.573.8133
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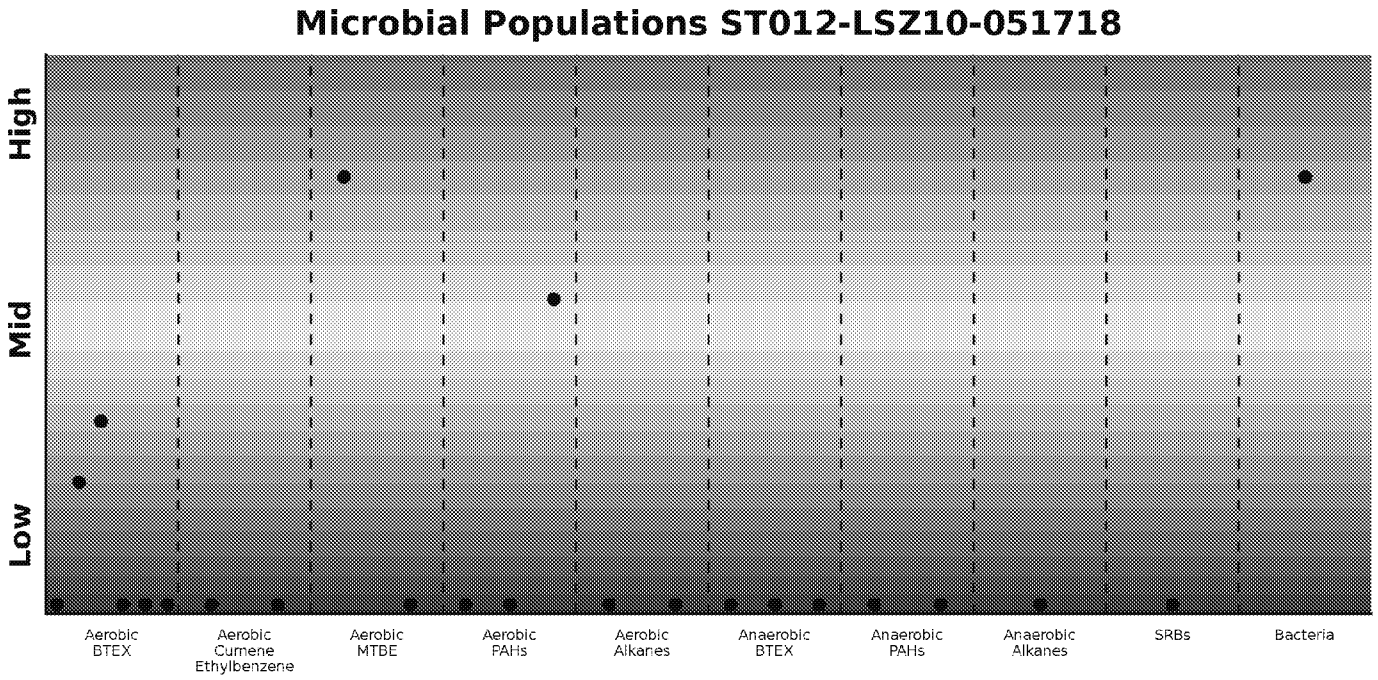

Figure 4: Microbial population summary to aid in evaluating potential pathways and biodegradation of specific contaminants.

Aerobic		Anaerobic	
BTEX	TOD, PHE, RDEG, RMO, TOL, EDO	BTEX	BCR, BSS, ABC
Cumene, Ethylbenzene	EDO, BPH4	Naphthalene/Methylnaphthalene	MNSSA, ANC
MTBE/TBA	PM1, TBA	Alkanes	assA
Naphthalene	NAH, NidA		
Phenanthrene	PHN		
Alkanes	ALK, ALMA		

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Knoxville, TN 37932
Phone: 865.573.8188
Fax: 865.573.8133
Web: www.microbe.com

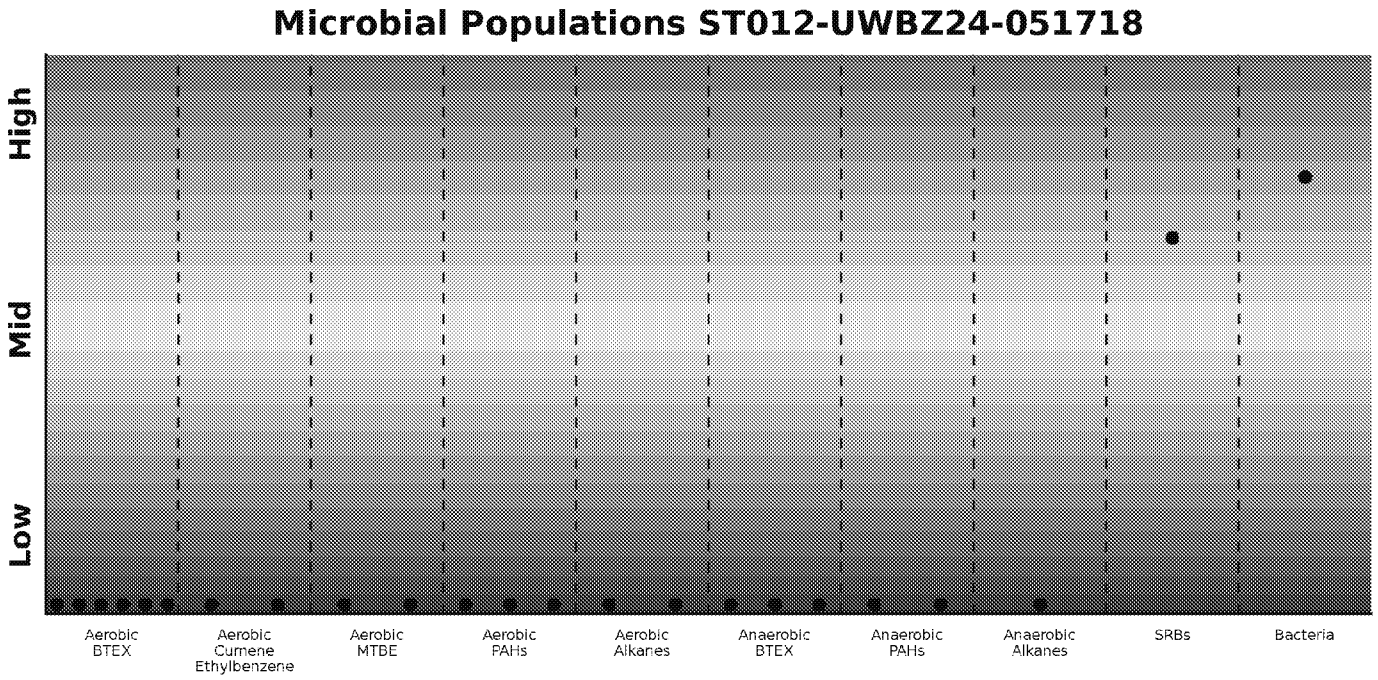

Figure 5: Microbial population summary to aid in evaluating potential pathways and biodegradation of specific contaminants.

Aerobic		Anaerobic	
BTEX	TOD, PHE, RDEG, RMO, TOL, EDO	BTEX	BCR, BSS, ABC
Cumene, Ethylbenzene	EDO, BPH4	Naphthalene/Methylnaphthalene	MNSSA, ANC
MTBE/TBA	PM1, TBA	Alkanes	assA
Naphthalene	NAH, NidA		
Phenanthrene	PHN		
Alkanes	ALK, ALMA		

10515 Research Drive
Knoxville, TN 37932
Phone: 865.573.8188
Fax: 865.573.8133
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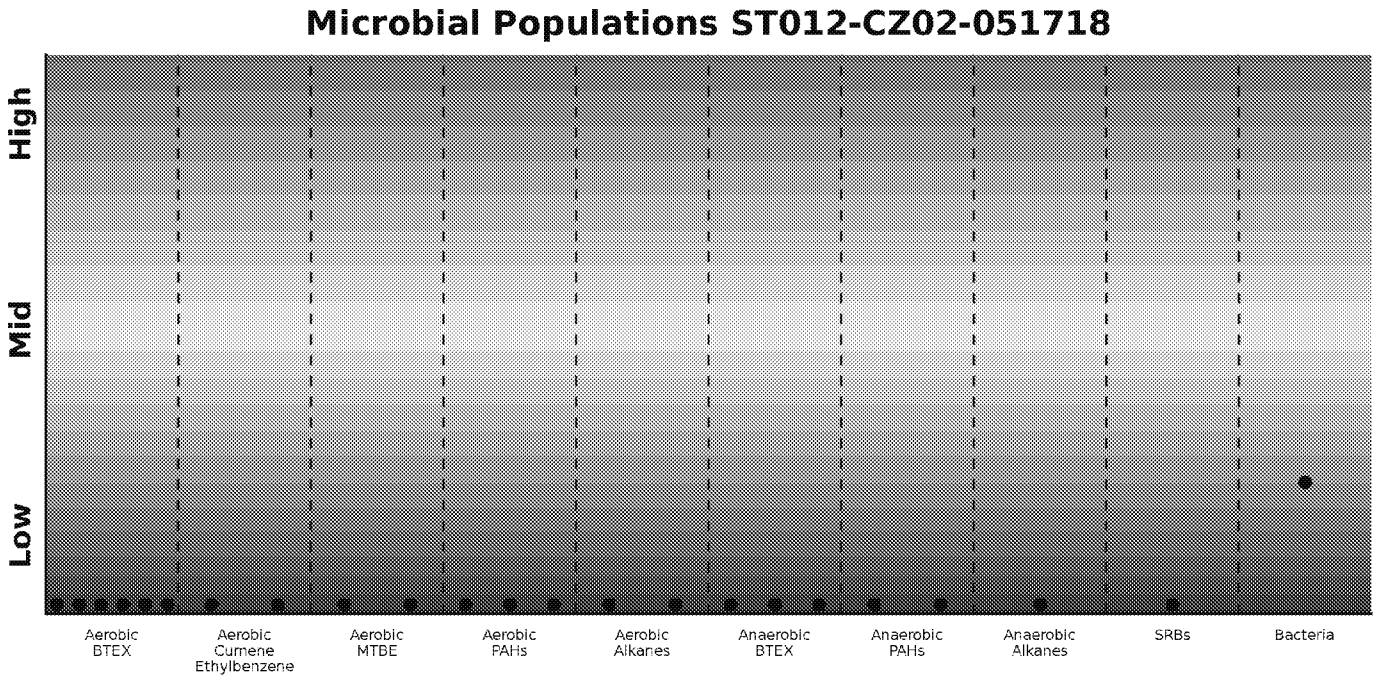

Figure 6: Microbial population summary to aid in evaluating potential pathways and biodegradation of specific contaminants.

Aerobic		Anaerobic	
BTEX	TOD, PHE, RDEG, RMO, TOL, EDO	BTEX	BCR, BSS, ABC
Cumene, Ethylbenzene	EDO, BPH4	Naphthalene/Methylnaphthalene	MNSSA, ANC
MTBE/TBA	PM1, TBA	Alkanes	assA
Naphthalene	NAH, NidA		
Phenanthrene	PHN		
Alkanes	ALK, ALMA		

10515 Research Drive
Knoxville, TN 37932
Phone: 865.573.8188
Fax: 865.573.8133
Web: www.microbe.com

Table 3: Summary of the QuantArray®-Petro results for microorganisms responsible for aerobic biodegradation of BTEX and MTBE for samples ST012-CZ20-051718, ST012-LSZ42-051718, and ST012-UWBZ31-051718.

Sample Name Sample Date	ST012-CZ20-051718 05/17/2018	ST012-LSZ42-051718 05/17/2018	ST012-UWBZ31-051718 05/17/2018
Aerobic BTEX and MTBE	cells/bead	cells/bead	cells/bead
Toluene/Benzene Dioxygenase (TOD)	**4.92E+02**	<2.50E+02	<2.50E+02
Phenol Hydroxylase (PHE)	**2.75E+03**	<2.50E+02	<2.50E+02
Toluene 2 Monooxygenase/Phenol Hydroxylase (RDEG)	**1.38E+05**	<2.50E+02	<2.50E+02
Toluene Ring Hydroxylating Monooxygenases (RMO)	<2.50E+02	<2.50E+02	<2.50E+02
Xylene/Toluene Monooxygenase (TOL)	<2.50E+02	<2.50E+02	<2.50E+02
Ethylbenzene/Isopropylbenzene Dioxygenase (EDO)	<2.50E+02	<2.50E+02	<2.50E+02
Biphenyl/Isopropylbenzene Dioxygenase (BPH4)	<2.50E+02	<2.50E+02	<2.50E+02
Methylibium petroleiphilum PM1 (PM1)	**2.50E+05**	<2.50E+02	<2.50E+02
TBA Monooxygenase (TBA)	<2.50E+02	<2.50E+02	<2.50E+02

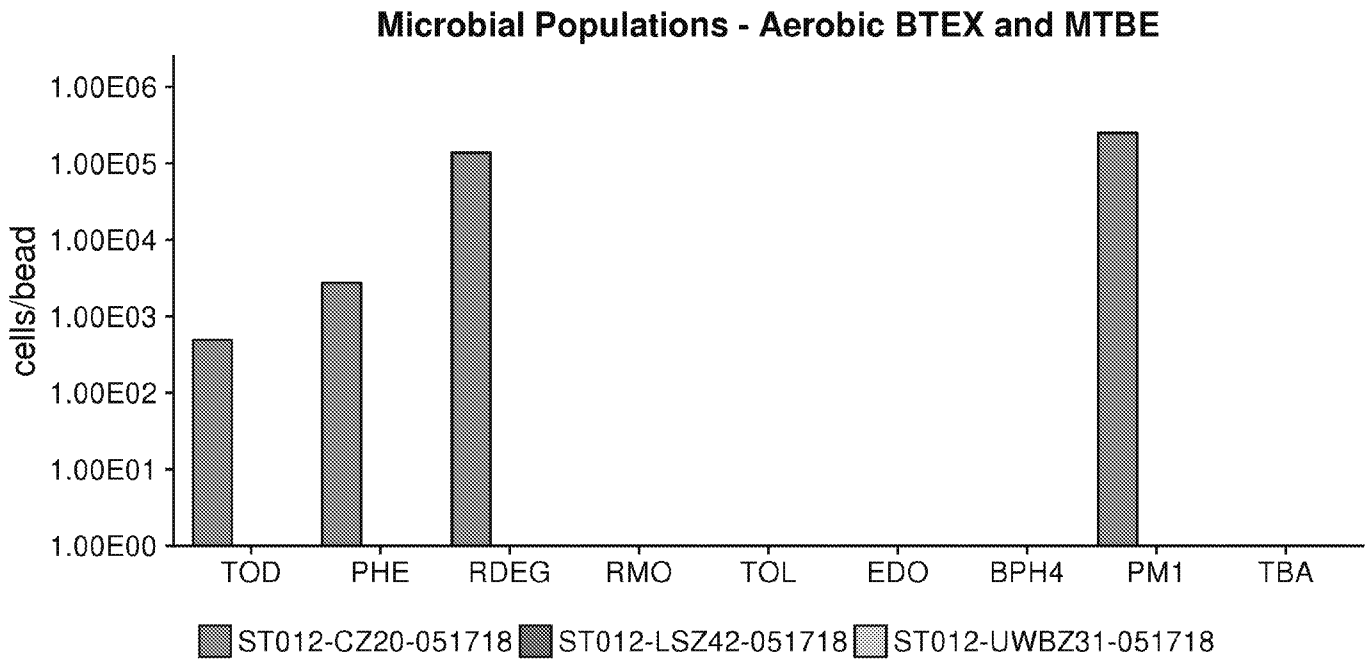

Figure 7: Comparison - microbial populations involved in aerobic biodegradation of BTEX and MTBE.

10515 Research Drive
Knoxville, TN 37932
Phone: 865.573.8188
Fax: 865.573.8133
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Table 4: Summary of the QuantArray®-Petro results for microorganisms responsible for aerobic biodegradation of BTEX and MTBE for samples ST012-LSZ10-051718, ST012-UWBZ24-051718, and ST012-CZ02-051718.

Sample Name Sample Date	ST012-LSZ10-051718 05/17/2018	ST012-UWBZ24-051718 05/17/2018	ST012-CZ02-051718 05/17/2018
Aerobic BTEX and MTBE	cells/bead	cells/bead	cells/bead
Toluene/Benzene Dioxygenase (TOD)	<2.50E+02	<2.50E+02	<2.50E+02
Phenol Hydroxylase (PHE)	**7.99E+02**	<2.50E+02	<2.50E+02
Toluene 2 Monooxygenase/Phenol Hydroxylase (RDEG)	**3.31E+03**	<2.50E+02	<2.50E+02
Toluene Ring Hydroxylating Monooxygenases (RMO)	<2.50E+02	<2.50E+02	<2.50E+02
Xylene/Toluene Monooxygenase (TOL)	<2.50E+02	<2.50E+02	<2.50E+02
Ethylbenzene/Isopropylbenzene Dioxygenase (EDO)	<2.50E+02	<2.50E+02	<2.50E+02
Biphenyl/Isopropylbenzene Dioxygenase (BPH4)	<2.50E+02	<2.50E+02	<2.50E+02
Methylibium petroleiphilum PM1 (PM1)	**1.03E+05**	<2.50E+02	<2.50E+02
TBA Monooxygenase (TBA)	<2.50E+02	<2.50E+02	<2.50E+02

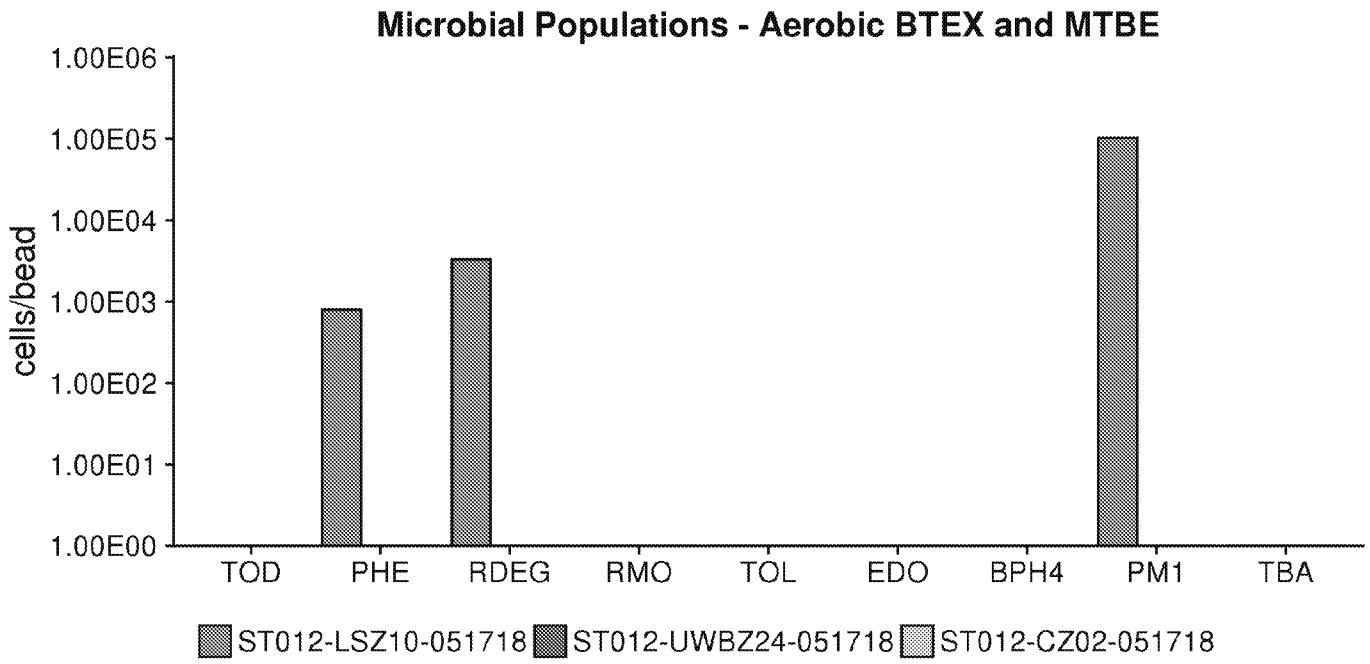

Figure 8: Comparison - microbial populations involved in aerobic biodegradation of BTEX and MTBE.

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Table 5: Summary of the QuantArray®-Petro results for microorganisms responsible for aerobic biodegradation of PAHs and alkanes for samples ST012-CZ20-051718, ST012-LSZ42-051718, and ST012-UWBZ31-051718.

Sample Name Sample Date	ST012-CZ20-051718 05/17/2018	ST012-LSZ42-051718 05/17/2018	ST012-UWBZ31-051718 05/17/2018
Aerobic PAHs and Alkanes	cells/bead	cells/bead	cells/bead
Naphthalene Dioxygenase (NAH)	<2.50E+02	<2.50E+02	<2.50E+02
Naphthalene-inducible Dioxygenase (NidA)	<2.50E+02	<2.50E+02	<2.50E+02
Phenanthrene Dioxygenase (PHN)	<2.50E+02	<2.50E+02	<2.50E+02
Alkane Monooxygenase (ALK)	<2.50E+02	<2.50E+02	<2.50E+02
Alkane Monooxygenase (ALMA)	<2.50E+02	<2.50E+02	<2.50E+02

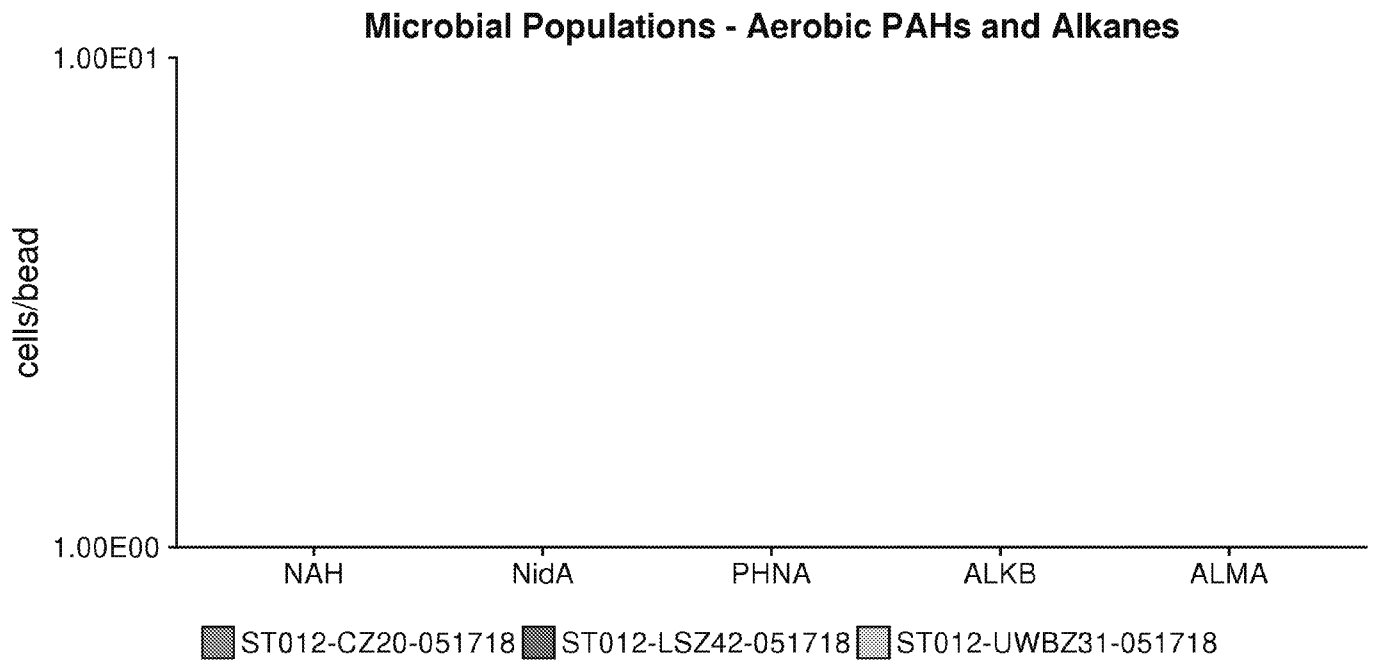

Figure 9: Comparison - microbial populations involved in aerobic biodegradation of PAHs and alkanes.

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Table 6: Summary of the QuantArray®-Petro results for microorganisms responsible for aerobic biodegradation of PAHs and alkanes for samples ST012-LSZ10-051718, ST012-UWBZ24-051718, and ST012-CZ02-051718.

Sample Name Sample Date	ST012-LSZ10-051718 05/17/2018	ST012-UWBZ24-051718 05/17/2018	ST012-CZ02-051718 05/17/2018
Aerobic PAHs and Alkanes	cells/bead	cells/bead	cells/bead
Naphthalene Dioxygenase (NAH)	**7.70E+00 (J)**	<2.50E+02	<2.50E+02
Naphthalene-inducible Dioxygenase (NidA)	<2.50E+02	<2.50E+02	<2.50E+02
Phenanthrene Dioxygenase (PHN)	**1.31E+03**	<2.50E+02	<2.50E+02
Alkane Monooxygenase (ALK)	<2.50E+02	<2.50E+02	<2.50E+02
Alkane Monooxygenase (ALMA)	<2.50E+02	<2.50E+02	<2.50E+02

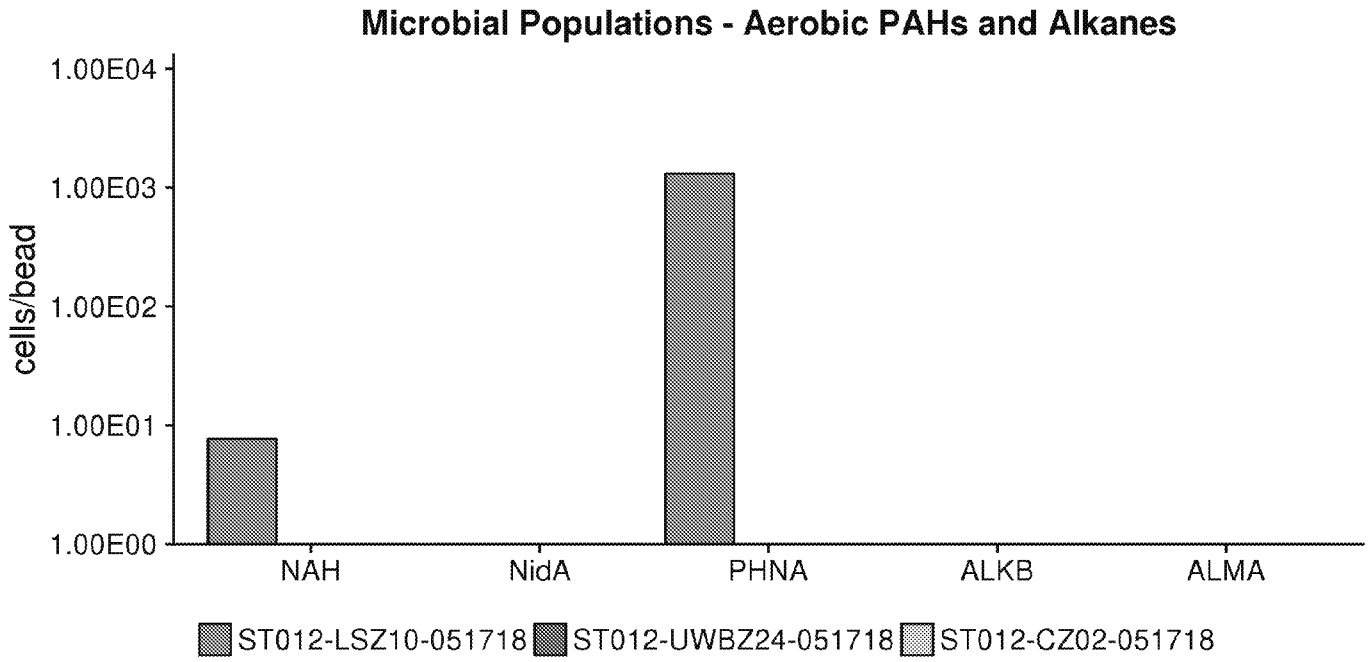

Figure 10: Comparison - microbial populations involved in aerobic biodegradation of PAHs and alkanes.

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Table 7: Summary of the QuantArray®-Petro results for microorganisms responsible for anaerobic biodegradation of BTEX, PAHs and alkanes for samples ST012-CZ20-051718, ST012-LSZ42-051718, and ST012-UWBZ31-051718.

Sample Name Sample Date	ST012-CZ20-051718 05/17/2018	ST012-LSZ42-051718 05/17/2018	ST012-UWBZ31-051718 05/17/2018
Anaerobic BTEX	cells/bead	cells/bead	cells/bead
Benzoyl Coenzyme A Reductase (BCR)	**4.57E+03**	<2.50E+02	<2.50E+02
Benzylsuccinate Synthase (BSS)	<2.50E+02	<2.50E+02	<2.50E+02
Benzene Carboxylase (ABC)	<2.50E+02	<2.50E+02	<2.50E+02
Anaerobic PAHs and Alkanes			
Naphthylmethylsuccinate Synthase (MNSSA)	<2.50E+02	<2.50E+02	<2.50E+02
Naphthalene Carboxylase (ANC)	<2.50E+02	<2.50E+02	<2.50E+02
Alkylsuccinate Synthase (ASS)	**4.20E+03**	<2.50E+02	<2.50E+02

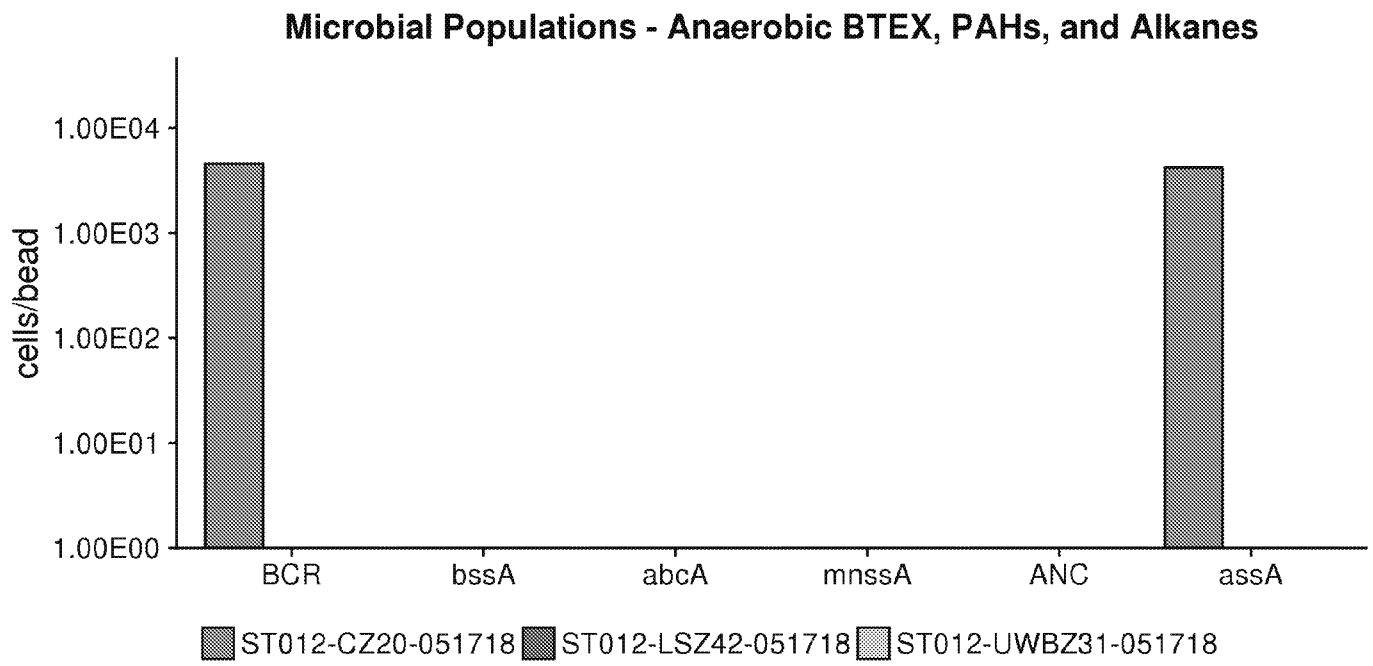

Figure 11: Comparison - microbial populations involved in anaerobic biodegradation of BTEX, PAHs and alkanes.

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Table 8: Summary of the QuantArray®-Petro results for microorganisms responsible for anaerobic biodegradation of BTEX, PAHs and alkanes for samples ST012-LSZ10-051718, ST012-UWBZ24-051718, and ST012-CZ02-051718.

Sample Name Sample Date	ST012-LSZ10-051718 05/17/2018	ST012-UWBZ24-051718 05/17/2018	ST012-CZ02-051718 05/17/2018
Anaerobic BTEX	cells/bead	cells/bead	cells/bead
Benzoyl Coenzyme A Reductase (BCR)	<2.50E+02	<2.50E+02	<2.50E+02
Benzylsuccinate Synthase (BSS)	<2.50E+02	<2.50E+02	<2.50E+02
Benzene Carboxylase (ABC)	<2.50E+02	<2.50E+02	<2.50E+02
Anaerobic PAHs and Alkanes			
Naphthylmethylsuccinate Synthase (MNSSA)	<2.50E+02	<2.50E+02	<2.50E+02
Naphthalene Carboxylase (ANC)	<2.50E+02	<2.50E+02	<2.50E+02
Alkylsuccinate Synthase (ASS)	<2.50E+02	<2.50E+02	<2.50E+02

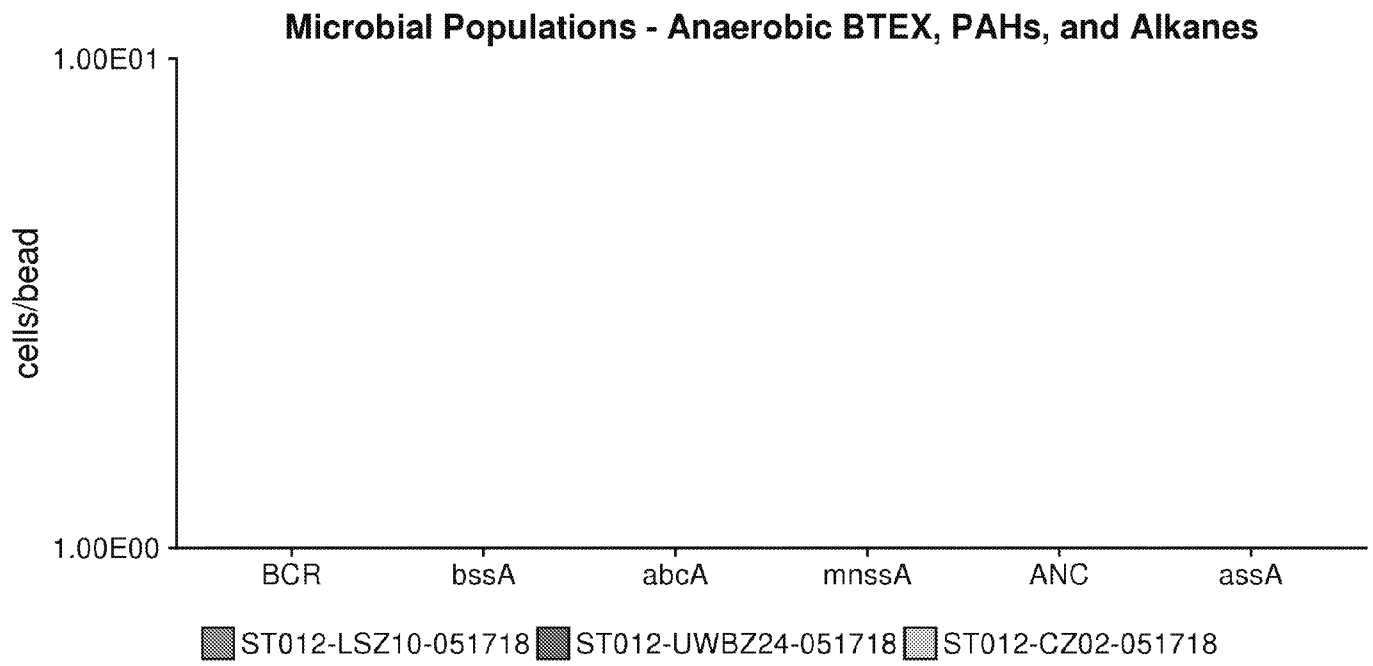

Figure 12: Comparison - microbial populations involved in anaerobic biodegradation of BTEX, PAHs and alkanes.

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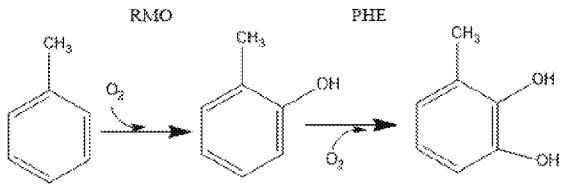
Interpretation

The overall purpose of the QuantArray®-Petro is to give site managers the ability to simultaneously yet economically evaluate the potential for biodegradation of a spectrum of contaminants found in petroleum products through a multitude of aerobic and anaerobic pathways to give a much more clear and comprehensive view of contaminant biodegradation. The following discussion describes interpretation of results in general terms and is meant to serve as a guide.

Aerobic Biodegradation - Benzene Toluene, Ethylbenzene, and Xylenes (BTEX): At sites impacted by petroleum products, aromatic hydrocarbons including BTEX are often contaminants of concern. Aerobic biodegradation of aromatic hydrocarbons has been intensively studied and multiple catabolic pathways have been well characterized. The substrate specificity of each pathway (range of compounds biodegraded via each pathway) is largely determined by the specificity of the initial oxygenase enzyme. The QuantArray®-Petro includes a suite of assays targeting the initial oxygenase genes of the known pathways for aerobic BTEX biodegradation.

Toluene/Benzene Dioxygenase (TOD): Toluene/benzene dioxygenase (TOD) incorporates both atoms of molecular oxygen into the aromatic ring. Although commonly called toluene dioxygenase, the substrate specificity of this enzyme is relaxed, allowing growth on toluene and benzene along with co-oxidation of a variety of compounds including ethylbenzene, *o*-xylene, *m*-xylene, and trichloroethene (TCE) when expressed.

Toluene/Benzene Monooxygenases (RMO/RDEG) and Phenol Hydroxylases (PHE): The next three known pathways for aerobic biodegradation of toluene (as well as benzene and xylenes) involve two steps: (1) an initial oxidation mediated by a toluene monooxygenase and (2) a second oxidation step catalyzed by a phenol hydroxylase. In these pathways, the toluene monooxygenases have been referred to as "ring hydroxylating monooxygenases" because they initiate biodegradation of toluene by incorporating oxygen directly into the aromatic ring rather than at a methyl group. The ring hydroxylating monooxygenases (RMOs) can be further described as toluene-2-monooxygenases, toluene-3-monooxygenases, or toluene-4-monooxygenases based upon where they attack the aromatic ring.



In General, phenol hydroxylases (PHE) catalyze the continued oxidation of phenols produced by RMOs. However, the difference between toluene monooxygenases (RMOs) and phenol hydroxylases (PHEs) is not absolute in terms of substrate specificity and catabolic function. For example, the TbmD toluene/benzene-2-monooxygenase [1] may be responsible for both the initial and second oxidation step [2].

The RMO, RDEG, and PHE assays target groups of genes encoding enzymes which perform the critical first and/or second steps in the aerobic biodegradation of BTEX compounds. In general terms, the RMO assay quantifies families of toluene-3-monooxygenase and toluene-4-monooxygenase genes. The RDEG assay is used to quantify groups of toluene-2-monooxygenase and phenol hydroxylase genes. Similarly, the PHE assay targets phenol hydroxylase genes and several benzene monooxygenase genes which catalyze both oxidation steps.

Toluene/Xylene Monooxygenase (TOL): The final known pathway for aerobic toluene biodegradation involves initial monooxygenase attack at the methyl group by a toluene/xylene monooxygenase.

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Ethylbenzene Dioxygenase (EDO): Similar to TOD, this group of aromatic oxygenases exhibits relatively broad specificity and is responsible for aerobic biodegradation of alkylbenzenes including ethylbenzene and isopropylbenzene or cumene [3].

Biphenyl Dioxygenase (BPH4): In environmental restoration, biphenyl dioxygenases are best known for cometabolism of polychlorinated biphenyls (PCBs). However, this subfamily includes benzene [4] and isopropylbenzene [5] dioxygenases from *Rhodococcus* spp.

Aerobic Biodegradation - MTBE and TBA: With increased use in the 1990s, the fuel oxygenate methyl *tert*-butyl ether (MTBE) has become one of the most commonly detected groundwater contaminants at gasoline contaminated sites. Pure cultures capable of utilizing MTBE as a growth supporting substrate have been isolated [6] and aerobic biodegradation of MTBE and the intermediate *tert*-butyl alcohol (TBA) has been reasonably well characterized. The QuantArray®-Petro includes quantification of two gene targets to assess the potential for aerobic biodegradation of MTBE and TBA.

Methylibium petroleiphilum PM1 (PM1): One of the few organisms isolated to date which is capable of utilizing MTBE and TBA as growth supporting substrates [6].

TBA Monooxygenase (TBA): Targets the TBA monooxygenase gene responsible for oxidation of TBA by *Methylibium petroleiphilum* PM1 [7].

Aerobic Biodegradation - Naphthalene and Other PAHs:

Naphthalene Dioxygenase (NAH): Naphthalene dioxygenase incorporates both atoms of molecular oxygen into naphthalene to initiate aerobic metabolism of the compound. However, the broad substrate specificity of naphthalene dioxygenase has been widely noted. When expressed, naphthalene dioxygenase is capable of catalyzing the oxidation of larger PAHs like anthracene, phenanthrene, acenaphthylene, fluorene, and acenaphthene. For a more comprehensive list of reactions mediated by naphthalene dioxygenases, see the University of Minnesota Biocatalysis/Biodegradation Database. (http://eawag-bbd.ethz.ch/naph/ndo.html, [8]).

Phenanthrene Dioxygenases (PHN): The PHN assays quantify phenanthrene/naphthalene dioxygenase genes from a diverse collection of microorganisms including *Pseudomonas*, *Burkholderia*, *Sphingomonas*, and *Acidovorax* spp. As with other naphthalene dioxygenases, substrate specificity is relatively broad and phenanthrene dioxygenases have been implicated in the biodegradation of naphthalene, phenanthrene, and anthracene and the co-oxidation of larger PAHs. Moreover, at least one research group has suggested that the PHN group of phenanthrene/naphthalene dioxygenases may be more environmentally relevant than the classical *nah*-like naphthalene dioxygenase [9].

Aerobic Biodegradation - *n*-alkanes: The *n*-alkanes are a substantial portion of petroleum products and are a component of TPH concentrations. The QuantArray®-Petro also includes quantification of alkane monooxygenase genes (ALK) which allow a wide range of *Proteobacteria* and *Actinomycetals* to grow on *n*-alkanes with carbon lengths from C_5 to C_{16} [10]. The QuantArray®-Petro also includes a second type of alkane hydroxylase (almA) which catalyzes the aerobic biodegradation of longer chain alkanes (C_{20}-C_{32}) by some *Alcanivorax* spp. considered dominant in marine systems [11].

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Anaerobic Biodegradation - Benzene, Toluene, Ethylbenzene, and Xylenes (BTEX): BTEX compounds are also susceptible to biodegradation under anoxic and anaerobic conditions although biodegradation pathways for each compound are not as well characterized as aerobic pathways. The QuantArray®-Petro includes sets of assays targeting a number of upper and lower pathway functional genes involved in the anaerobic catabolism of BTEX compounds for better evaluation of anaerobic biodegradation at petroleum contaminated sites.

Benzylsuccinate Synthase (BSS): Of the BTEX compounds, toluene biodegradation under anaerobic conditions is the most extensively studied and best characterized. The first step in this pathway, mediated by benzylsuccinate synthase (*bss*A) is the addition of fumarate onto the toluene methyl group to form benzylsuccinate. While additional pathways are possible, some bacterial isolates capable of anaerobic biodegradation of ethylbenzene and xylenes follow the same metabolic approach where the first step is the addition of fumarate.

Anaerobic Benzene Carboxylase (ABC): Although additional pathways are possible, the only pathway for anaerobic biodegradation of benzene elucidated to date is initiated by a benzene carboxylase enzyme.

Benzoyl Coenzyme A Reductase (BCR): Benzoyl-CoA is the central intermediate in the anaerobic biodegradation of many aromatic hydrocarbons. Benzoyl-CoA Reductase (BCR) is the essential enzyme for reducing the benzene ring structure.

Anaerobic Biodegradation - PAHs: The anaerobic biodegradation of PAHs involves analogous mechanisms to those described for anaerobic biodegradation of BTEX compounds. For example, the anaerobic biodegradation of methyl-substituted PAHs like 2-methylnaphthalene is initiated by fumarate addition to the methyl group while the only characterized pathway for anaerobic naphthalene biodegradation is initiated by a carboxylase.

Naphthylmethylsuccinate Synthase (MNSSA): MNSSA is analogous to the benzylsuccinate synthase described above for anaerobic biodegradation of toluene. Naphthylmethylsuccinate synthase catalyzes the addition of fumarate onto the methyl group of 2-methylnaphthalene [12].

Anaerobic Naphthalene Carboxylase (ANC): To date, the only pathway that has been characterized for anaerobic biodegradation of naphthalene is initiated by a naphthalene carboxylase enzyme [13].

Anaerobic Biodegradation - *n*-alkanes: As mentioned previously, the *n*-alkanes are a substantial portion of petroleum products and should be considered particularly when site cleanup goals include TPH reduction. The addition of fumarate is a common mechanism for activating and initiating biodegradation of a variety of petroleum hydrocarbons under anaerobic conditions including *n*-alkanes. The QuantArray®-Petro includes quantification of alkyl succinate synthase genes (assA) which have been characterized in nitrate reducing and sulfate reducing isolates utilizing *n*-alkanes from C_6 to at least C_{18} [14].

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